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Bimatoprost and prostaglandin $F_{2\alpha}$ selectively stimulate intracellular calcium signaling in different cat iris sphincter cells

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Abstract

Bimatoprost is a synthetic analog of prostaglandin $F_{2\alpha}$ ethanolamide (prostamide $F_{2\alpha}$), and shares a pharmacological profile consistent with that of the prostamides. Like prostaglandin $F_{2\alpha}$ carboxylic acid, bimatoprost potently lowers intraocular pressure in dogs, primates and humans. In order to distinguish its mechanism of action from prostaglandin $F_{2\alpha}$, fluorescence confocal microscopy was used to examine the effects of bimatoprost, prostaglandin $F_{2\alpha}$ and 17-phenyl prostaglandin $F_{2\alpha}$ on calcium signaling in resident cells of digested cat iris sphincter, a tissue which exhibits contractile responses to both agonists. Constant superfusion conditions obviated effective conversion of bimatoprost. Serial challenge with 100 nM bimatoprost and prostaglandin $F_{2\alpha}$ consistently evoked responses in different cells within the same tissue preparation, whereas prostaglandin $F_{2\alpha}$ and 17-phenyl prostaglandin $F_{2\alpha}$ elicited signaling responses in the same cells. Bimatoprost-sensitive cells were consistently re-stimulated with bimatoprost only, and prostaglandin $F_{2\alpha}$ sensitive cells could only be re-stimulated with prostaglandin $F_{2\alpha}$. The selective stimulation of different cells in the same cat iris sphincter preparation by bimatoprost and prostaglandin $F_{2\alpha}$, along with the complete absence of observed instances in which the same cells respond to both agonists, strongly suggests the involvement of distinct receptors for prostaglandin $F_{2\alpha}$ and bimatoprost. Further, prostaglandin $F_{2\alpha}$ but not bimatoprost potently stimulated calcium signaling in isolated human embryonic kidney cells stably transfected with the feline- and human-prostaglandin $F_{2\alpha}$ FP-receptor and in human dermal fibroblast cells, and only prostaglandin $F_{2\alpha}$ competed with radioligand binding in HEK-feFP cells. These studies provide further evidence for the existence of a bimatoprost-sensitive receptor that is distinct from any of the known prostaglandin receptor types.

Keywords: bimatoprost; calcium signaling; cat iris sphincter; prostaglandin $F_{2\alpha}$; 17-phenyl prostaglandin $F_{2\alpha}$

1. Introduction

The prostaglandins (PGs) are naturally occurring oxygenated arachidonic acid metabolites which are endogenously produced via cyclo-oxygenase-1 (COX-1) and COX-2 enzymatic pathways (Bazan and Allan, 1997; Dubois et al., 1998; Vane et al., 1998). PGs exert a diverse range of biological activities through interaction with discrete receptor binding sites that are present in virtually every vertebrate tissue (Coleman et al., 1990, 1994). In the eye, PGs have been shown to decrease intraocular pressure (IOP) by increasing uveoscleral outflow through various postulated mechanisms, including relaxation of the ciliary muscle or remodeling of the extracellular matrix in the ciliary body (Crawford et al., 1987; Gabelt and Kaufman, 1989, 1990; Krauss et al., 1997; Nilsson et al., 1989; Schachtschabel et al., 2000; Toris et al., 1995; Weinreb et al., 2002). Synthetic derivatives of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) have been successfully developed into clinically acceptable topical therapies for the management of increased IOP (Camras et al., 1996; Sorbera and Castaner, 2000; Taniguchi et al., 1996).

The prostamides are recently discovered biologically active substances that are derived from anandamide, a fatty

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acid amide precursor that serves as a substrate for the COX-2 enzyme (Burstein et al., 2000; Di Marzo, 1998; Kozak et al., 2001; Yu et al., 1997), and have recently been shown to be biosynthesized in vivo (Weber et al., 2004). Moreover, prostamides appear to possess a unique pharmacological profile and are distinct from PGs in that they do not meaningfully interact with any of the known PG-receptors (Berglund et al., 1999; Matias et al., 2004; Ross et al., 2002; Woodward et al., 2000). Whereas the pharmacologic characterization of the PG-receptor family into DP-, EP-, FP-, IP- and TP-receptor subtypes has been supported in its entirety by molecular biological studies, and most proposed PG-receptor subtypes have been cloned (Coleman, 1998; Woodward et al., 1997), prostamide receptors have only recently been proposed based on pharmacological criteria.

Bimatoprost is a synthetically derived analog of prostaglandin $F_{2\alpha}$ ethanolamide (prostamide $F_{2\alpha}$). It potently decreases IOP in dogs and primates, and has proven to be a highly effective ocular hypotensive agent in humans (Brubaker et al., 2001; Cantor, 2001; DuBiner et al., 2001; Easthope and Perry, 2002; Gandolfi et al., 2001; Sherwood and Brandt, 2001; Woodward et al., 2001, 2003). Binding studies performed on a wide array of receptors, ion channels and transporters have demonstrated that bimatoprost does not meaningfully interact with adrenergic, cholinergic, cannabinoid, dopaminergic, or any of the known prostaglandin receptors ($IC_{50} > 10\,000$ nM), indicating that these receptors are not involved in mediation of bimatoprost-induced responses (Woodward et al., 2001). Bimatoprost exhibited similar potency to $PGF_{2\alpha}$, however, in one preparation containing FP-receptors: the cat iris sphincter (Woodward et al., 2001).

In order to elucidate whether the mechanism of action for bimatoprost involves the prostaglandin FP-receptor or, alternatively, a distinct receptor site in this preparation, fluorescence confocal microscopy was used to examine the effects of bimatoprost, $PGF_{2\alpha}$ and 17-phenyl $PGF_{2\alpha}$ on calcium (Ca^{2+}) signaling. Rapid superfusion conditions were chosen so that potential hydrolysis of the amide to the free acid could not affect Ca^{2+} signals in the cells of cat iris sphincter preparations, a tissue in which bimatoprost exhibits similar degrees of contractile potency to that of $PGF_{2\alpha}$. For this reason, comparison of $PGF_{2\alpha}$, 17-phenyl $PGF_{2\alpha}$ and bimatoprost effects in isolated cat iris sphincter cells provides a highly discriminating test for the concept that prostaglandin-sensitive FP-receptors and prostamide-sensitive receptors are distinct. Further agonist activities of bimatoprost, $PGF_{2\alpha}$ and 17-phenyl $PGF_{2\alpha}$ on Ca^{2+} signaling were examined in human embryonic kidney cells stably expressing the feline FP-receptor (HEK-feFP) and in human dermal fibroblast cells, which constitutively express the FP-receptor. The effects of bimatoprost and 17-phenyl $PGF_{2\alpha}$ on Ca^{2+} signaling were also examined in HEK cells stably expressing the human FP receptor (HEK-huFP). Competitive binding using [^{3}H]-17-phenyl $PGF_{2\alpha}$ versus unlabeled bimatoprost and 17-phenyl $PGF_{2\alpha}$ was also examined in HEK-feFP cells. The combined data show that there is no overlap in the pharmacological activity of bimatoprost and $PGF_{2\alpha}$, thereby demonstrating that bimatoprost interacts with an as yet undefined receptor.

2. Materials and methods

2.1. Drug solutions

Bimatoprost (Synthetic Chemistry, Allergan, Inc., Irvine, CA), [^{3}H]-17-phenyl $PGF_{2\alpha}$ (Amersham Pharmacia Biotech, Piscataway, NJ), 17-phenyl $PGF_{2\alpha}$ and $PGF_{2\alpha}$, (Cayman Chemical Co., Ann Arbor, MI) were chemically pure. The amide bimatoprost as well as $PGF_{2\alpha}$ acid were prepared as 1×10^{-3} and 1×10^{-4} M stock solutions, the former by solubilizing in ethanol, and the latter by solubilizing in 2% Na_2CO_3, and subsequently bringing to volume in Tris buffered 0·1% physiological saline solution. Carbachol (Sigma Chemical Co., Inc., St Louis, MO) was prepared as a 1×10^{-3} M stock solution in deionized H_2O. Stock solutions were stored at 4°C. Working solutions of control and test substances were freshly prepared immediately prior to use as 1:1000 dilutions of stock preparations (v/v) in physiological medium.

2.2. Time-resolved confocal imaging of Ca^{2+} signaling in cat iris sphincter tissue

2.2.1. Tissue digestion

Animal studies were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the US National Institutes of Health. Iris sphincter tissues were obtained from freshly enucleated eyes of adult domestic female cats, which were euthanised by intravenous Eutha-6 CII (Western Medical Supply Co., Arcadia, CA). Tissue specimens were obtained by first removing the cornea and then transecting the iris into quadrants, which were kept in place by the intact underlying lens/vitreous body. Tissue specimens were removed by carefully cutting narrow strips from the distal tip of the iris forming the boundary of the pupillary opening; the iris sphincter can be visually distinguished by its slightly different coloration and reduced thickness relative to the bulk of the iridial tissue. Tissues were pooled in 1× Dulbecco's Modified Eagle Medium, low glucose and without glutamine or foetal bovine serum/bovine serum albumin (Gibco BRL/Life Technologies, Rockville, MD) and subsequently minced into smaller pieces and washed by gravity in two changes of 1 ml of physiological medium containing 2 mg ml^{-1} bovine serum albumin (BSA) to remove excess free melanin. The washed tissue was first digested in 20 ml of 0·75 mg ml^{-1} collagenase B (Boehringer Mannheim, Mannheim, Germany) in physiological medium with BSA for 20 min at 37°C with constant agitation., washed in one change of 1 ml physiological

medium with BSA, and subsequently digested in 20 ml of 0·5 mg ml^{-1} collagenase B/0·35 mg ml^{-1} pronase (Sigma Chemical Co., Inc., St Louis, MO) in physiological medium with BSA for 20 min at 37°C with constant agitation. The digested tissue was washed once with 1 ml physiological medium with BSA, resuspended in 1 ml physiological medium with BSA and then loaded with 10 μM Fluo-4 AM dye (Molecular Probes, Inc., Eugene, OR) for 30 min at 37°C. Following Fluo-4 loading, the tissue fragments were resuspended in 1 ml physiological medium without BSA.

2.2.2. Confocal microscopy under superfusion conditions

25 mm round glass coverslips prepared on the day to be used by coating with a solution of 1·5:1 Cell-Tak (Collaborative Biomedical Products, Bedford, MA):0·1 M $NaHCO_3$ and allowed to air-dry at 37°C were subsequently placed in a two-piece metal chamber fitted for a flow-through stage micro-incubator (model PDMI-2, Harvard Apparatus, Holliston, MA) maintained at 37°C with a bipolar/monopolar temperature controller unit (model TC-202A, Harvard Apparatus, Holliston, MA). Digested tissue fragments suspended in a minimal volume (~3–5 μl) of physiological medium without BSA were allowed to settle onto the heated coverslips for approximately 10 min so that they would adhere to the Cell-Tak coating, after which the chamber was superfused with physiological medium without BSA at an approximate flow rate of 0·6 ml min^{-1}. Flow/aspiration was maintained by a peristaltic pump (Dynamax RP-1, Rainin Instrument Co., Inc., Woburn, MA) at a rate of four changes of chamber volume per minute.

Acquisition of images in cat iris sphincter tissue preparations was performed with a Zeiss LSM 510 confocal microscope workstation employing argon laser excitation at the 488 nm line, and 510–570 nm emission filtration. Control and test substances were perfused into and aspirated from the chamber via a peristaltic pump. Physiological medium without BSA was perfused prior to administration of bimatoprost and $PGF_{2\alpha}$ Following superfusion of bimatoprost, $PGF_{2\alpha}$, or 17-phenyl $PGF_{2\alpha}$, the chamber was perfused with physiological medium with BSA without drugs for several minutes to expedite the washout of these lipophilic substances, followed by a several-minute wash with physiological medium without BSA. The order in which the carbachol control, bimatoprost, $PGF_{2\alpha}$, or 17-phenyl $PGF_{2\alpha}$ were administered was randomly altered from experiment to experiment to ensure that responses were not affected by sensitisation or desensitisation to control or test substances.

Quantitative region-of-interest (ROI) analysis was performed with the Zeiss LSM confocal workstation software after thorough review of the entire field to locate areas exhibiting responses which correlated with application of the compounds. The ROI data was exported as ASCII flat-files and imported into PSI-Plot (Poly Software International, Pearl River, NY) for graphical representation of signal tracings for the entire course of each experiment. In a number of later experiments, serial confocal optical sections of ROIs were acquired immediately after fields for Ca^{2+} signaling analysis had been collected in order to enable construction of three-dimensional projections using the confocal workstation software. *z*-axis resolutions for the projections were generally in the range of 0·2–0·6 μm, depending on the extent of sample thickness as determined by minimum and maximum confocal limits within which any objects in the ROI remained visible. Responding cells were identified in the three-dimensional projections by correlating the locations of drug-related responses in fields from the time-based Ca^{2+} signaling series with the identical locations in the respective serial optical sections used to generate the projection. Because representation of time-based changes in three-dimensional reconstructions was precluded by the confocal software, selected views captured as two-dimensional image frames from projections rotated about the *y*-axis were exported into Adobe Photoshop (Adobe Systems, Inc., San Jose, CA) so that responding cells could be indicated in the captured image frames with superimposed graphics.

2.3. Agonist-induced contraction of isolated cat iris sphincter tissue

Iris sphincter tissues were obtained from freshly enucleated eyes of adult domestic cats as described above under the methods for time-resolved confocal imaging of Ca^{2+} signaling. Iris sphincter smooth muscle tissue specimens from each eye were dissected into two strips, and the tissue suspended with suture at each end in 10-ml jacketed organ baths containing Krebs buffer with 1 μM indomethacin (Sigma Chemical Co., Inc., St Louis, MO), maintained at 37°C and continuously bubbled with a 95% O_2/5% CO_2 gas mixture to sustain a pH of 7·4. Smooth muscle tension was measured isometrically with force displacement transducers and recorded on a Grass Model 7 polygraph (Grass Instrument Co., Inc., Quincy, MA). The tissues were equilibrated for 60 min under 50–100 mg tension. Different concentrations of test compounds were cumulatively added to the organ bath. The contractile response to 10^{-7} M $PGF_{2\alpha}$ was determined at the beginning and end of each experiment. The activity of compounds was expressed as a percentage of the 10^{-7} M $PGF_{2\alpha}$-induced reference contraction.

2.4. Preparation of HEK-293 cells stably transfected with the recombinant feline (HEK-feFP) or human (HEK-huFP) FP-receptors

HEK-293 cells were grown in Dulbecco's Modified Eagle Medium (DMEM)/10% fetal bovine serum (FBS) plus 250 μg ml^{-1} G418 and 200 μg ml^{-1}

penicillin/streptomycin (Invitrogen Life Technologies, Carlsbad, CA). For transfection, cells were grown to 50–60% confluency on 10 cm plates. 20 μg pCEP4 plasmid incorporating cDNA inserts for the feline or the human FP-receptor was added to 500 μg of 250 mM $CaCl_2$. 2×HEPES-buffered saline (2×HBS; 280 mM NaCl, 20 mM HEPES acid, 1·5 mM Na_2HPO_4, pH 7·05–7·12) was then added with continuous vortexing at room temperature dropwise to make a total of 500 μl. After 30 min, a 9-ml volume of DMEM was added to the mixture. The DNA/DMEM/$CaCl_2$ mixture was then added to the cells, which had been previously rinsed with 10 ml phosphate-buffered saline (PBS). The cells were incubated at 37°C for 5 h in a humidified atmosphere of 95% air/5% CO_2. The $CaCl_2$ solution was removed and the cells treated with 10% lipofectamine in DMEM for 2 min. The lipofectin solution was replaced with DMEM/10% FBS and the cells incubated overnight, after which the incubation medium was replaced with DMEM/10% FBS containing 250 μg ml^{-1} G418 and 200 μg ml^{-1} penicillin/streptomycin, followed by another 24-hr incubation period. Hygromycin B was then added to a final concentration of 200 μg ml^{-1} and hygromycin-resistant clones selected eight days later. Each clone was transferred to separate wells on a 24-well plate, and grown to confluence, and then transferred into separate 25-ml culture flasks. Cells were maintained under continuous hygromycin selection until use.

2.5. Radioligand binding to the recombinant feline FP-receptor in HEK-feFP cells

2.5.1. Preparation of cell membrane fraction

HEK-feFP cells were harvested at 70–100% confluency and washed in 50 mM TME buffer (50 mM Tris base, 10 mM $MgCl_2$, 1 mM EDTA, pH 7·4) at 4°C. Cells were homogenized in ice-cold TME buffer at a setting of 7 for 30 sec with a polytron (Brinkman PT 10/35, Brinkman Instruments, Inc., Westbury, NY). Buffer was added as necessary to maintain a 50-ml volume in the centrifuge tubes. The homogenate was pelleted by centrifugation at 19 000 rpm for 20 min at 4–6°C using a Beckman Ti-60 rotor (Beckman–Coulter, Inc., Fullerton, CA). The pellet was re-homogenized in cold TME buffer at setting four for about 5 sec, and resuspended in fresh TME buffer to provide a final protein concentration of 1 mg ml^{-1}, as determined by Biorad assay.

2.5.2. Radioligand binding assay

The binding of 5 nM [^{3}H]-17-phenyl $PGF_{2\alpha}$ (85 Ci $mmol^{-1}$, specific activity) was determined in duplicate against unlabeled bimatoprost and 17-phenyl $PGF_{2\alpha}$, with each experiment replicated three times. Incubations were done in a final volume of 100 μl for 60 min at 25°C. Binding reactions were initiated by adding plasma membrane fraction and terminated by the addition of 4 ml ice-cold 10 mM Tris/HCl buffer, pH 7·4, followed by rapid filtration through glass fiber GF/B filters (Whatman, Maidstone, UK) and three additional washes in a cell harvester (Brandel Medical Research and Development Laboratories, Inc., Gathersburg, MD). The filters were oven-dried for 1 hr and counting performed with a Beckman–Coulter LS 6500 Multi-Purpose Scintillation Counter (Beckman–Coulter, Inc., Fullerton, CA).

2.6. Ca^{2+} signaling measured by fluorescence spectrophotometry in HEK-feFP, HEK-huFP and human dermal fibroblast cells

HEK-feFP or HEK-huFP stable transfectant cells were harvested at 70–90% confluency from culture flasks by scraping. Confluent human dermal CRL 1497 fibroblast cells grown in DMEM/10% FBS containing 250 μg ml^{-1} G418 and 200 μg ml^{-1} penicillin/streptomycin, under a humidified atmosphere of 95% air/5% CO_2 at 37°C, were harvested from culture flasks by treatment with 0·05% trypsin/0·52 mM EDTA (Gibco, Grand Island, NY) at 37°C for approximately 1 min; proteolytic activity was arrested by the addition of 5 ml FBS in DMEM. Cells were consecutively washed in Hank's basic saline solution (BSS) and medium containing 140 mM NaCl, 50 mM KCl, 1 mM $MgCl_2$, 1·5 mM $CaCl_2$, 10 mM HEPES and TRIS, 5 mM glucose, 5 mM Na pyruvate, 0·1% bovine serum albumin (BSA), pH 7·4. Centrifugation for the washes was performed at 200×g for 15 min at room temperature. Cells were counted, resuspended in the medium detailed above and incubated for 30 min with 2×10^{-6} M Fura 2/acetoxymethyl ester (Molecular Probes, Inc., Eugene, OR) in a shaker water bath maintained at 37°C. The cells were again washed with the medium above, and resuspended at a concentration of 2×10^6 cells ml^{-1}, and 0·5-ml aliquots added to autocap microtubes to provide 10^6 cells per experimental determination of intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$).

Fluorescence was measured in a Perkin–Elmer LS-50 B luminescence spectrophotometer (Perkin–Elmer, Norwalk, CT) at excitation and emission wavelengths of 340 and 492 nm, respectively, with both slits set at a bandwidth of 10 nm. For each experimental determination, 10^6 cells were washed by centrifugation at 200×g for 5 min and suspended in a 2-ml cuvette with buffer containing 120 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1·5 mM $CaCl_2$, 20 mM HEPES, 1 mg ml^{-1} glucose and 1 mg ml^{-1} Na pyruvate. Cells were kept in suspension with a magnetic stirrer, and temperature was maintained at 37°C. Calibration of the Fura 2 signal was performed as previously described; cells were lysed with 10 μl of 100 mg ml^{-1} digitonin in DMSO to obtain f_{max} and 100 mM EGTA with sufficient NaOH to adjust the pH to 8·5 successively added to obtain f_{min}.

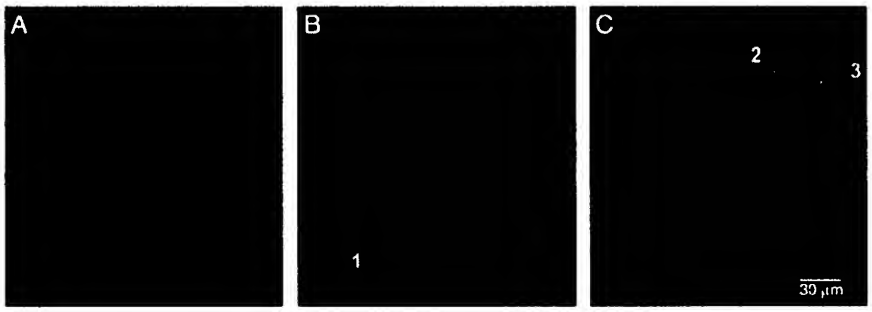

Fig. 1. Fluorescent confocal images of the same region-of-interest (ROI) showing selective calcium (Ca^{2+}) signaling in bimatoprost-sensitive (1) and $PGF_{2\alpha}$-sensitive (2, 3) cells in a cat iris sphincter tissue digest preparation under constant perfusion with buffered medium only (A), buffered medium with 100 nM bimatoprost (B), and buffered medium with 100 nM $PGF_{2\alpha}$. The ROI shown was extracted from a field observed under 20× magnification.

3. Results

3.1. Ca^{2+} signaling—confocal imaging of cat iris sphincter tissue digests

100 nM or 1 μM of bimatoprost or $PGF_{2\alpha}$ consistently evoked Ca^{2+} signaling responses in different cells within the same cat iris sphincter tissue digest preparation (Fig. 1). In general, an estimated 10–15% of cells included within the acquired field responded to $PGF_{2\alpha}$, whereas bimatoprost typically evoked responses in less than 10% of observed cells. Moreover, changing the order in which the test compounds were applied did not result in any observed instances in which bimatoprost and $PGF_{2\alpha}$ evoked responses in the same cell (Fig. 2). Sequential challenge with both agonists in the same tissue preparation did not result in any observed instances in which the same cell responded to both bimatoprost and $PGF_{2\alpha}$; randomly altering the order of bimatoprost and $PGF_{2\alpha}$ challenge provided no evidence of desensitisation or priming of responses in the same cell

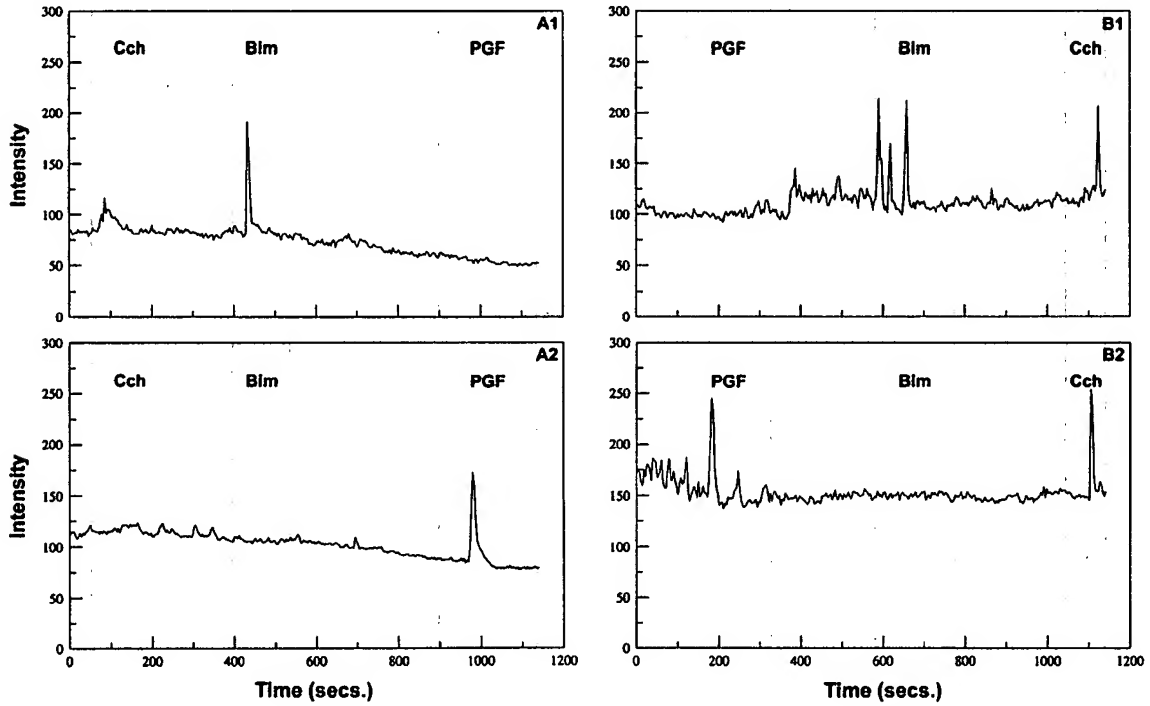

Fig. 2. Representative examples of Ca^{2+} recordings induced in cat iris sphincter tissue digest preparations obtained under constant perfusion conditions following sequential challenge with bimatoprost (Bim; upper panel of each pair of graphs) and $PGF_{2\alpha}$ (PGF; lower panel of each pair of graphs), from the same ROI. Sequential dosing of the same tissue digest preparation with bimatoprost and $PGF_{2\alpha}$ selectively stimulates Ca^{2+} signals in different cells within the same cat iris sphincter tissue digest preparation. Altering the order in which agonists are administered does not affect the selectivity of cellular responses in the same tissue preparation. The shaded vertical bands denote the period of suffusion with the indicated agonist. In some cases, cells which responded to carbachol also exhibited responses to bimatoprost or $PGF_{2\alpha}$, the ratio of cells responding to both carbachol control and the respective test substance often varying significantly from one tissue mount preparation to another.

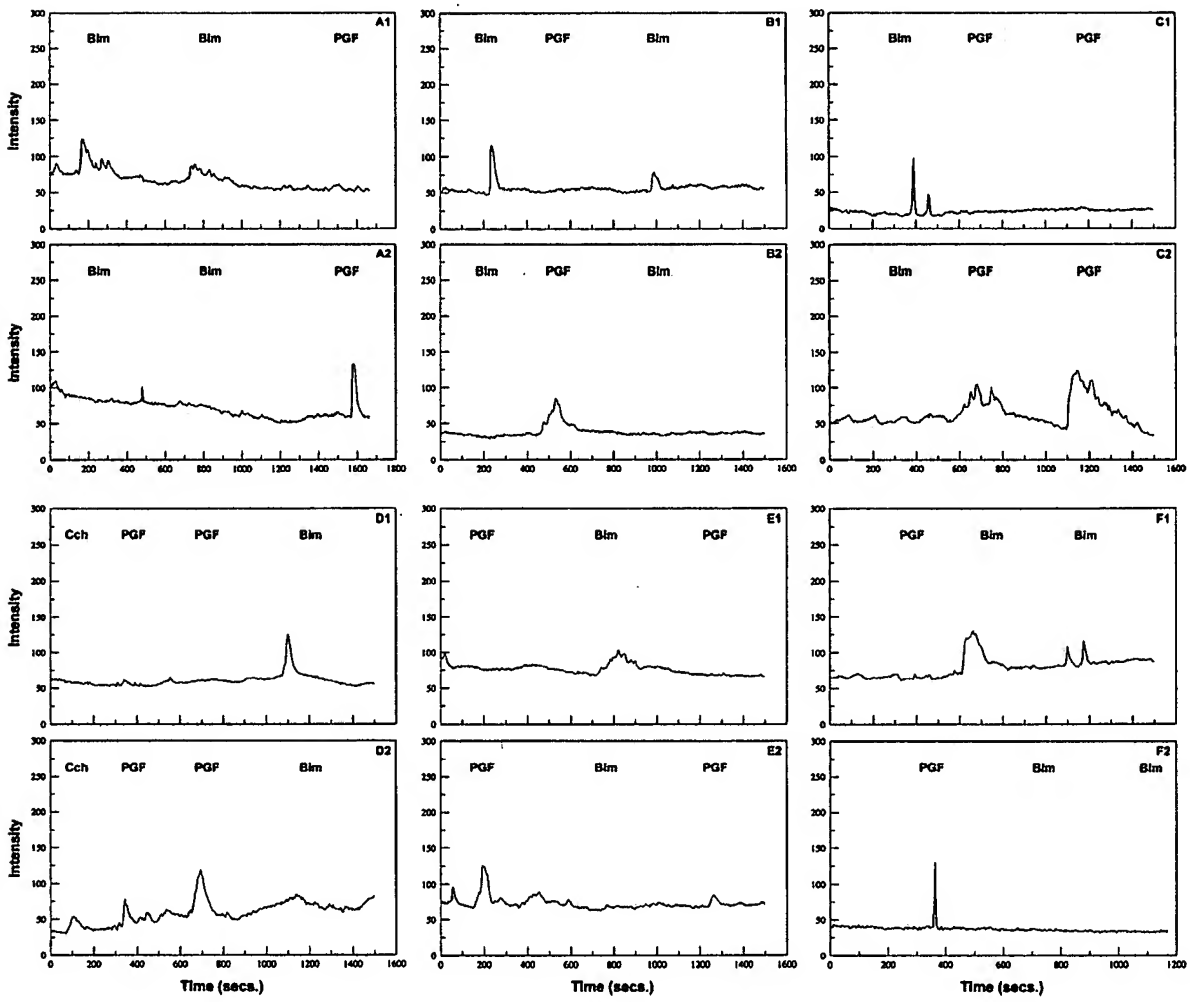

Fig. 3. Representative examples of Ca^{2+} recordings induced in cat iris sphincter tissue digest preparations obtained under constant perfusion conditions following sequential challenge with bimatoprost (Bim; upper panel of each pair of graphs) and $PGF_{2\alpha}$ (PGF; lower panel of each pair or graphs), from the same ROI, except the pair at the lower right (F1/F2), which shows results from two different coverslips prepared from the same tissue digest Repetitive dosing of the same tissue digest preparation with bimatoprost or $PGF_{2\alpha}$ selectively evokes Ca^{2+} signals in the same cell, whereas signaling responses are never induced in the same cell by both agonists. Altering the order in which the same cat iris sphincter preparation is sequentially challenged with bimatoprost and $PGF_{2\alpha}$ does not affect the selectivity of cellular responses in the same tissue preparation. The shaded vertical bands denote the period of suffusion with the indicated agonist. In some cases, cells which responded to carbachol also exhibited responses to bimatoprost or $PGF_{2\alpha}$, the ratio of cells responding to both carbachol control and the respective test substance often varying significantly from one tissue mount preparation to another. Carbachol was omitted in a number of later multiple or sequential challenge studies to allow for more thorough washing-out periods in order to avoid significantly increasing the overall duration of experiments and possible negative impacts on tissue viability.

(Fig. 3). Additionally, bimatoprost-sensitive cells did not respond to either $PGF_{2\alpha}$ or 17-phenyl-$PGF_{2\alpha}$ in tissue preparations sequentially challenged with bimatoprost, $PGF_{2\alpha}$ and 17-phenyl $PGF_{2\alpha}$; in contrast, $PGF_{2\alpha}$ and 17-phenyl $PGF_{2\alpha}$ consistently evoked signaling in the same cells (Fig. 4). Signal amplitudes induced in the same cell by the same agonist were not always similar; in some cases, the amplitude of the initial response was less than that of the subsequent response, and in other instances the amplitude of the second response was not as great as that of the first. These observations were made in a total of 62 iris sphincter tissue digest preparations from different individual animals, and 2–4 replicate studies performed with each tissue digest preparation.

Carbachol-induced signaling was observed in an estimated 25–40% of observed cells, depending on the individual tissue preparation. In some cases, cells which responded to carbachol also exhibited responses to bimatoprost or $PGF_{2\alpha}$, the ratio of cells responding to both carbachol control and the respective test substance often varying significantly from one tissue mount preparation to another. Intrinsic background Ca^{2+} signaling was largely suppressed by 100 nM atropine included in working solutions of bimatoprost or $PGF_{2\alpha}$, thereby facilitating the distinction of specific signaling induced by control and/or test compounds from spontaneous activity during review of the animated image sequences. Bimatoprost, $PGF_{2\alpha}$, and 17-phenyl $PGF_{2\alpha}$, as well as carbachol, occasionally

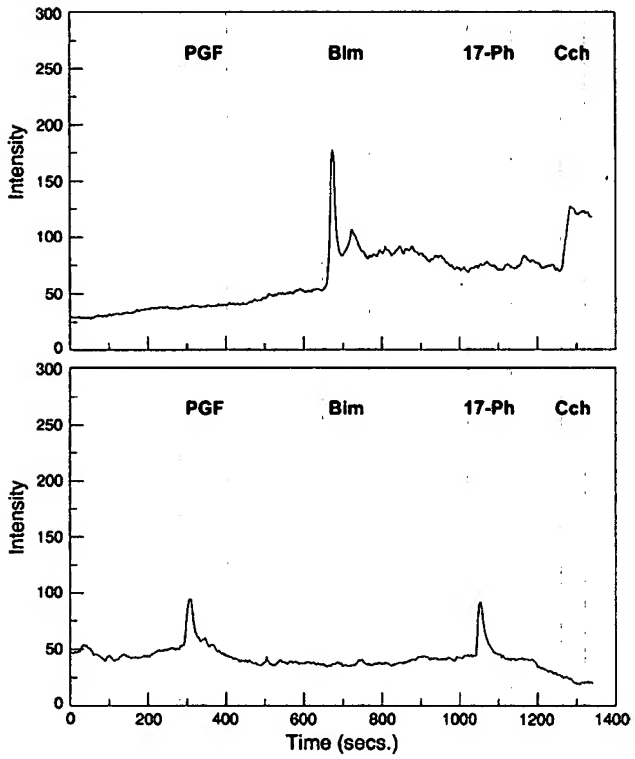

Fig. 4. A representative example of Ca^{2+} recordings induced in cat iris sphincter tissue digest preparations obtained under constant perfusion conditions following sequential challenge with bimatoprost (Bim; upper panel), $PGF_{2\alpha}$ and 17-phenyl $PGF_{2\alpha}$ (PGF, 17-Ph, respectively; lower panel), from the same ROI. $PGF_{2\alpha}$ and 17-phenyl-$PGF_{2\alpha}$ elicited signaling responses in the same cells, whereas Ca^{2+} signaling evoked by challenge with bimatoprost was observed in different cells in the same tissue preparation. Altering the order in which the same cat iris sphincter preparation is sequentially challenged with bimatoprost, $PGF_{2\alpha}$ and 17-phenyl $PGF_{2\alpha}$ does not affect the selectivity of cellular responses in the same tissue preparation (data not shown). The shaded vertical bands denote the period of suffusion with the indicated agonist. In some cases, cells which responded to carbachol also exhibited responses to bimatoprost or $PGF_{2\alpha}$, the ratio of cells responding to both carbachol control and the respective test substance often varying significantly from one tissue mount preparation to another. Carbachol was omitted in some studies to allow for more thorough washing-out periods in order to avoid significantly increasing the overall duration of experiments and possible negative impacts on tissue viability.

induced contraction of cat iris sphincter tissue digests that was significant enough to result in movement of the ROI. The atropine included in bimatoprost and $PGF_{2\alpha}$ working solutions had no observable effects on such contractile responses, whereas carbachol-induced contraction was abolished in the presence of atropine. Experiments in which significant ROI movement was observed were excluded from consideration.

Three-dimensional reconstructions obtained in a number of later experiments by serial confocal optical sectioning of ROIs immediately following acquisition of fields for the analysis of Ca^{2+} signaling revealed that bimatoprost-sensitive and $PGF_{2\alpha}$-sensitive cells appear to differ in their overall gross morphological characteristics (Fig. 5). While $PGF_{2\alpha}$-sensitive cells consistently exhibited an elongated form reminiscent of smooth-muscle cell contour, bimatoprost-sensitive cells were spheroidal in shape.

3.2. Contractile responses in isolated cat iris sphincter tissue

In the cat iris sphincter, $PGF_{2\alpha}$ and bimatoprost were similarly potent in their ability to induce contractile responses, with EC_{50} values of 10·1 and 29·7 nM, respectively. 17-phenyl $PGF_{2\alpha}$ exhibited an EC_{50} of 2·3 nM (Fig. 6).

3.3. Ca^{2+} signaling—fluorescence spectrophotometry

At the recombinant feline FP-receptor overexpressed in HEK cells, the EC_{50} values for 17-phenyl $PGF_{2\alpha}$, $PGF_{2\alpha}$, and bimatoprost were 2·4, 9·1 and >10 μM, respectively (Fig. 7). EC_{50} values for 17-phenyl $PGF_{2\alpha}$ and bimatoprost at the recombinant human FP-receptor in HEK-huFP cells were 1·5 and >10 μM, respectively (Fig. 8). In human dermal fibroblasts, 17-phenyl $PGF_{2\alpha}$ and $PGF_{2\alpha}$ exhibited EC_{50} values of 11·5 nm and 33·5 nM, respectively; the EC_{50} for bimatoprost exceeded 10 μM (Fig. 9). Thus, 17-phenyl $PGF_{2\alpha}$ and $PGF_{2\alpha}$ potently interacted with both recombinant and natural FP-receptors, whereas the effects of bimatoprost were not pharmacologically meaningful.

3.4. Radioligand binding

Radioligand binding competition with [^{3}H]-17-phenyl $PGF_{2\alpha}$ in HEK-feFP cells yielded an IC_{50} for bimatoprost of 8900 nM, in contrast to an IC_{50} value of 13·2 nM for 17-phenyl $PGF_{2\alpha}$ (Fig. 10). Specific binding obtained was approximately 93%.

4. Discussion

Previous comprehensive studies have shown that the pharmacological and functional characteristics of bimatoprost appear to be unrelated to activity at any of the PG-sensitive receptors (Woodward et al., 2001, 2003). The structural basis of bimatoprost's unique activity is due to the substitution of the negatively charged carboxylic acid group at the C-1 position with a neutral ethyl amide moiety. It is known that replacing the carboxylic acid component of $PGF_{2\alpha}$ with neutral alkoxy, amide, or hydroxyl substituents markedly reduces $PGF_{2\alpha}$-like agonist activity (Maddox et al., 1978; Schaaf and Hess, 1979; Woodward et al., 2000). The results we report herein lend further support to the contention that bimatoprost possesses a distinct pharmacological and functional profile and that its potent action is not mediated by interaction with PG-receptors or related to its conversion to the free acid.

The present studies demonstrate unequivocally that bimatoprost and $PGF_{2\alpha}$ selectively evoke Ca^{2+} signaling

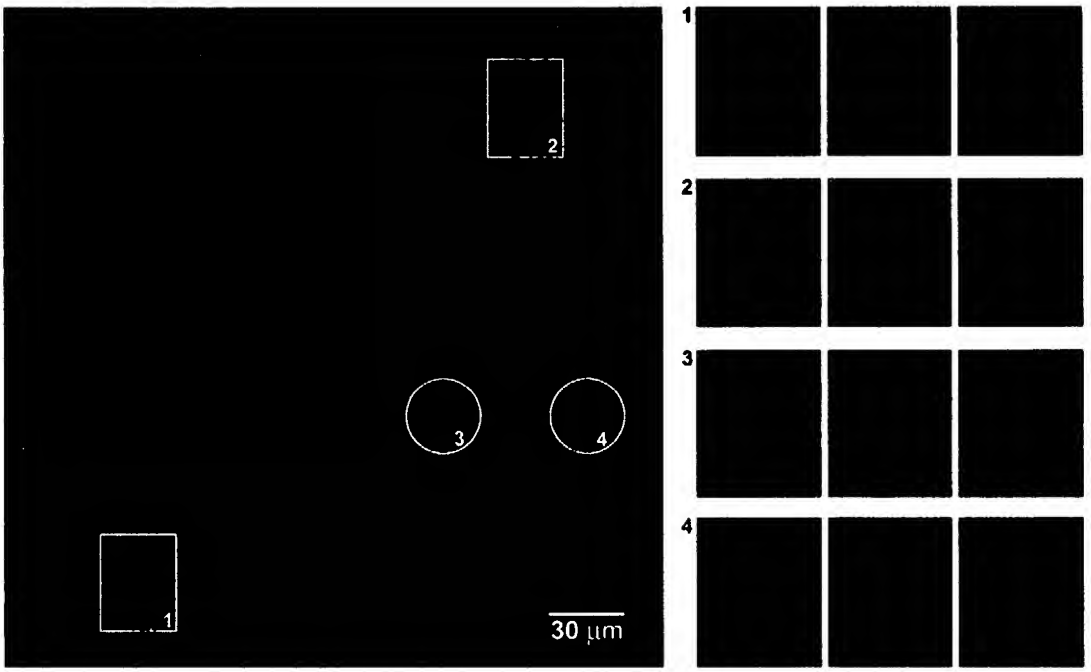

Fig. 5. A selected region of a representative ROI volumetrically reconstructed from serial confocal optical sections showing gross morphological characteristics of $PGF_{2\alpha}$-sensitive cells (1, 2; indicated by a rectangular marquee) and bimatoprost-sensitive cells (3, 4; indicated by a circular marquee). A series of panels on the right show enlarged images of corresponding cells numbered in the overall field on the left, viewed from different angles as the three-dimensional projection was rotated around the *y*-axis. The projection was constructed from optical sections obtained under 40× magnification.

in different cells within the same cat iris sphincter tissue preparation observed under constant-flow conditions. Cells sensitive to bimatoprost are reliably re-stimulated when challenged repetitively with 100 nM or 1 μM bimatoprost only, and $PGF_{2\alpha}$-sensitive cells consistently respond after repetitive challenge with the same concentrations of $PGF_{2\alpha}$ only. Cell selectivity of Ca^{2+} signal stimulation is also maintained following sequential challenge in the same tissue preparation by both agonists, with no indications that attenuation or augmentation of Ca^{2+} signaling responses result from prior challenge with either agonist. These observations, made in 62 different cat iris sphincter tissue digest preparations, clearly indicate that bimatoprost and $PGF_{2\alpha}$ are acting directly through different receptor sites.

Several reports maintain that bimatoprost functions as a prodrug, and that the active form arises through conversion to a free acid, the selective and potent FP-receptor agonist, 17-phenyl $PGF_{2\alpha}$ (Davies et al., 2003; Hellberg et al., 2003; Maxey, et al., 2002; Sharif et al., 2002). Although the continuous superfusion of buffer through the incubation chamber employed in our confocal studies would effectively preclude any realistic possibility that the observed cellular responses to either agonist could be ascribed to a metabolite of the parent compound, additional studies comparing the responses to sequential challenge with bimatoprost, $PGF_{2\alpha}$ and 17-phenyl $PGF_{2\alpha}$ in the same tissue preparation showed that bimatoprost-sensitive cells did not respond to either $PGF_{2\alpha}$ or 17-phenyl $PGF_{2\alpha}$, whereas $PGF_{2\alpha}$ and 17-phenyl $PGF_{2\alpha}$ consistently evoked Ca^{2+} signaling in the same cells. These observations are consistent with other work showing that prostamides do not undergo significant conversion to their corresponding PGs after prolonged incubation periods in various tissue homogenates (Matias et al., 2004), and that bimatoprost is the predominant molecular species present at all time points in

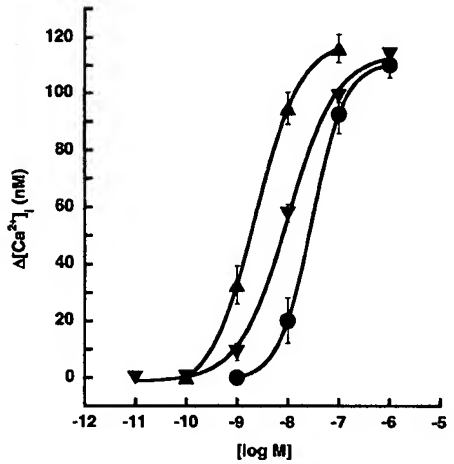

Fig. 6. The contractile effects of 17-phenyl-trinor-$PGF_{2\alpha}$ (▲), $PGF_{2\alpha}$ (▼) and bimatoprost (●) in feline iris sphincter. Contractile responses are expressed as percent of contraction induced by 10^{-7} M $PGF_{2\alpha}$. ($n=4$, 17-phenyl-trinor-$PGF_{2\alpha}$; $n=4$, $PGF_{2\alpha}$; $n=4$, bimatoprost).

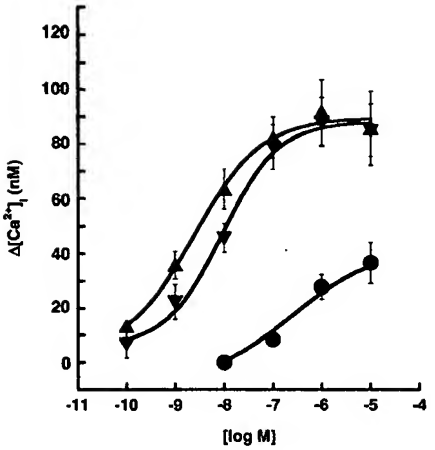

Fig. 7. The effects of 17-phenyl-trinor-$PGF_{2\alpha}$ (▲), $PGF_{2\alpha}$ (▼) and bimatoprost (●) on changes in intracellular Ca^{2+} concentration as measured by fluorescence spectrophotometry in HEK 293 cells stably expressing the feline prostaglandin FP receptor (HEK-feFP) ($n=3$).

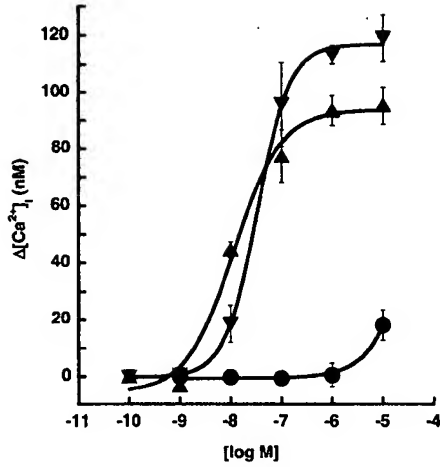

Fig. 9. The effects of 17-phenyl-trinor-$PGF_{2\alpha}$ (▲), $PGF_{2\alpha}$ (▼) and bimatoprost (●) on changes in intracellular Ca^{2+} concentration as measured by fluorescence spectrophotometry in human dermal fibroblast cells ($n=4$).

primate ocular tissues following topical application (Woodward et al., 2003). Given these results, it is difficult to imagine a scheme in which the bimatoprost parent compound ultimately exerts its effects via a $PGF_{2\alpha}$-sensitive receptor site.

The selective nature of the Ca^{2+} signaling responses evident in the confocal studies is also consistent with our results from functional studies in isolated HEK-feFP and HEK-huFP stably transfected cells, and in human dermal fibroblast cells, which constitutively express the FP-receptor. In all instances, bimatoprost is substantially and uniformly less potent than 17-phenyl-$PGF_{2\alpha}$ and $PGF_{2\alpha}$ in eliciting Ca^{2+} signaling, and that comparable ranking of potencies are demonstrated at both the feline and the human FP-receptors. It should be noted that these findings are in agreement with those of previous investigations showing that Ca^{2+} signaling is potently and reliably induced by $PGF_{2\alpha}$ in Swiss 3T3 cells, which express the FP-receptor, whereas bimatoprost fails to produce significant alteration of intracellular Ca^{2+} concentrations (Woodward et al., 2001). Another group of investigators has presented results where high concentrations of bimatoprost were claimed to displace $PGF_{2\alpha}$ from the FP receptor in bovine corpus luteum homogenates and also activate FP-receptors in Swiss 3T3 mouse fibroblasts and HEK cells stably transfected with cloned human ciliary body FP-receptors, as well as normal human trabecular meshwork cells (Sharif et al., 2001, 2002, 2003a,b). However, the concentrations required to elicit these responses were much higher than those required for

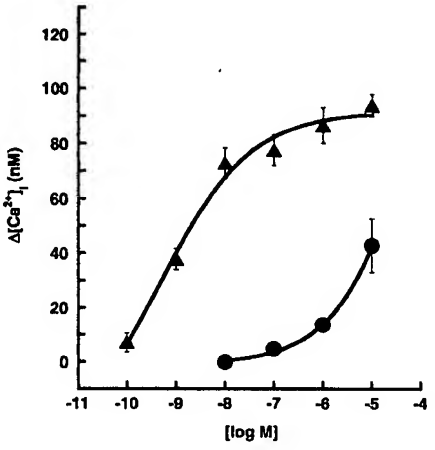

Fig. 8. The effects of 17-phenyl-trinor-$PGF_{2\alpha}$ (▲) and bimatoprost (●) on changes in intracellular Ca^{2+} concentration as measured by fluorescence spectrophotometry in HEK 293 cells stably expressing the human prostaglandin FP receptor (HEK-huFP). ($n=7$, 17-phenyl-trinor-$PGF_{2\alpha}$; $n=4$, bimatoprost).

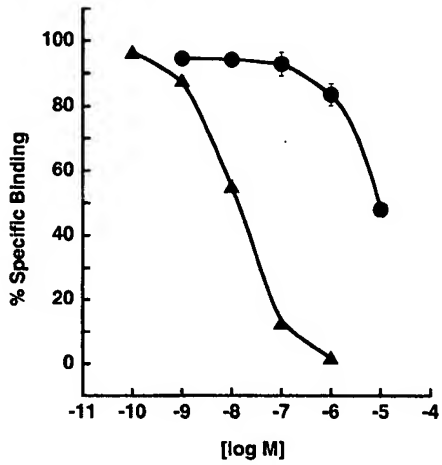

Fig. 10. Competitive displacement of [^{3}H]-17-phenyl-trinor-$PGF_{2\alpha}$ by 17-phenyl-trinor-$PGF_{2\alpha}$ (▲) and bimatoprost (●) at the recombinant feline prostaglandin FP receptor in HEK-feFP cells ($n=3$).

prostamide activity, indicating that the FP-receptor cannot be the primary target site for bimatoprost.

Isolated whole tissue studies have shown that bimatoprost and $PGF_{2\alpha}$ are equally potent in causing contraction of this tissue. Whether these contractile responses are mediated by coupling via the same or different pathways remains to be elucidated. However, the ability of bimatoprost and $PGF_{2\alpha}$ to cause contraction in iris sphincter tissue is not inconsistent with action through different receptors. The finding that atropine abolishes contraction elicited by carbachol in cat iris sphincter tissue digests while not affecting bimatoprost- or $PGF_{2\alpha}$-induced contraction indicates that different receptor subtypes can mediate contractile responses in the iris sphincter. Contraction has been presumed to be directly mediated by smooth muscle cells that are radially distributed in the iris sphincter. Longitudinal iridial dilator muscle elements are not implicated because they project from the region of the iris root and terminate approximately three-fourths of the distance into the iris leaf proper and can be clearly distinguished from the iris sphincter region upon removal of the pigmented epithelium. Only the iris sphincter was recovered for use in our tissue contraction and confocal studies. In this regard, the possibility that more than one cell type is involved in mediating contraction of the cat iris sphincter either through direct or indirect activity is suggested by the differential morphology of $PGF_{2\alpha}$-sensitive and bimatoprost-sensitive cells as shown in three-dimensional reconstructions of ROIs. A multiple-cell hypothesis is at this point speculative and additional work is required to elucidate whether such a mechanism of action operates in the iris sphincter.

The results of the studies, which we report herein expand upon previous investigations showing that bimatoprost exhibits no meaningful FP-receptor agonist activity or interaction with the FP-receptor. In particular, the consistent and selective induction of Ca^{2+} signaling in different cells within the same constant-flow cat iris sphincter tissue digest preparation by serial challenge with bimatoprost and $PGF_{2\alpha}$ strongly suggests the involvement of distinct receptor sites. Taken together, the results of our studies provide further evidence that bimatoprost potently stimulates receptor sites that are distinct from $PGF_{2\alpha}$-sensitive receptors.

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